B-4567
[1-7]

RESEARCH ARTICLE Indian Journal of Animal Research, Volume Issue : ()

Construction and Characterization of the NS3 Protein of Bluetongue Virus as A Bait for Application in Yeast Two-hybrid System

Ashwini R. Chaple[1], Gaurav K. Sharma[2], Udit Joshi[1], Dhanavelu Muthuchelvan[3], Pallavi Deol[2], Sonalika Mahajan[1]

10.18805/IJAR.B-4567

ABSTRACT

Background: Bluetongue is an arthropod transmitted viral disease causing range of clinical manifestations in small ruminants. The causative agent, Bluetongue virus (BTV) possesses several structural (SPs) and non-structural proteins (NSPs) associated with its morphogenesis and virulence. NS3, a multifunctional protein of BTV execute a decisive role in virus pathogenesis and release. In the present study, the NS3 protein of BTV was cloned into a pGBKT7-BD vector as bait and characterized for its expression. The suitability of NS3 as a bait to screen interactions with host proteins was also assessed.

Methods: The NS3 gene was cloned in the pGBKT7-BD vector to generate the bait plasmid. The recombinant pGBKT7-NS3 bait was sequence confirmed and characterized for auto-activation, toxicity and expression. Thereafter, the bait was mated with yeast two-hybrid (Y2H) cDNA library and screened for the host-BTV protein-protein interactions (PPI).

Conclusion: The constructed bait was found suitable for mating, however, no protein hits appeared on forward library screening. The sequence analysis revealed the presence of the transmembrane domains (TM) within the NS3 sequence that may have resulted in the failure of protein interaction studies in Y2H.

Key words: Bait, cDNA library, Cytotoxicity, NS3 protein, Yeast two-hybrid.

INTRODUCTION

Bluetongue (BT) is an infectious and devastating viral disease of domestic and wild ruminants. The disease poses significant losses to livestock health and the agricultural economy due to high morbidity, mortality, abortion, teratogenicity, reduced performance and restrictions on livestock trade (Sperlova and Zendulkova, 2011; Saminathan *et al*. 2020). It is primarily an arthropod-transmitted disease, other modes of transmission can occur rarely (Mellor, 1990; Meiswinkel *et al*. 2008; Belbis *et al*. 2013; Coetzee *et al*. 2012). BT has been reported from several parts of the world and has become endemic in Indian subcontinents (Mellor *et al*. 2008; Sreenivasulu *et al*. 2004; Rao *et al*. 2016). The clinical manifestation is characterized by fever, facial edema, hemorrhages, oral mucosal ulceration and coronitis. Although the clinical presentation of BT varies widely among different ruminant hosts, sheep and white-tailed deer show the lethal form of the disease (Modrow *et al*. 2010; Rojas *et al*. 2019).

The etiological agent of BT is the Bluetongue virus (BTV), a non-enveloped, double-stranded RNA (dsRNA) virus with ten segments. It belongs to the genus *Orbivirus* within the family *Reoviridae*. To date, 27 distinct serotypes of BTV are reported worldwide (Alkhamis *et al*. 2020) along with the two putative serotypes *i.e*., 28 and 29 (Bumbarov *et al*. 2020; Yang *et al*. 2020). BTV genome is approximately 19.2 kb which encodes seven SPs (VP1-VP7) and five NSPs (NS1-NS5) (Belhouchet *et al*. 2011; Stewart *et al*. 2015; Ratinier *et al*. 2011). These SPs and NSPs play a diverse function in virus morphogenesis and pathogenicity (Schwartz-Cornil *et al*. 2008).

NS3 protein of BTV executes multiple functions within-host including virus release by viroporin formation. It is encoded by segment 10 and occurs in two isoforms *i.e.* NS3 and NS3A (Celma and Roy 2009; Han and Harty 2004). Albeit, the individual role of NS3A has not been identified yet, NS3/NS3A is the only membrane glycoprotein synthesized by BTV (Wu *et al*. 1992). The NS3 protein sequence is remarkably conserved among the various BTV strains and is considered a potent candidate for diagnostic

[1]Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute, Izatnagar-243 122, Uttar Pradesh, India.
[2]Centre for Animal Disease Research and Diagnosis, ICAR-Indian Veterinary Research Institute, Izatnagar-243 122, Uttar Pradesh, India.
[3]Division of Veterinary Virology, ICAR-Indian Veterinary Research Institute, Bengaluru-560 064, Karnataka, India.

Corresponding Author: Sonalika Mahajan, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute, Izatnagar-243 122, Uttar Pradesh, India. Email: sonalikam1@gmail.com

How to cite this article: Chaple, A.R., Sharma, G.K., Joshi, U., Muthuchelvan, D., Deol, P. and Mahajan, S. (2021). Construction and Characterization of the NS3 Protein of Bluetongue Virus as A Bait for Application in Yeast Two-hybrid System. Indian Journal of Animal Research.DOI:10.18805/IJAR.B-4567

Submitted: 09-06-2021 **Accepted:** 24-07-2021 **Online:** 09-08-2021

purposes. Despite these facts, detailed studies are not available on the host-pathogenesis aspect of NS3. Thus the present study is designed to explore the interaction of NS3 protein with host proteins employing the yeast two-hybrid (Y2H) system. Y2H system is a dynamic technique to study the PPI based on a realignment of functional transcription factor (TF) during the interaction of two proteins of interest (Fields and Song 1989). cDNA library from sheep origin was used as prey to investigate the protein-protein interaction (PPI) with BTV NS3 bait.

MATERIALS AND METHODS

Cells and virus

BTV serotype 10 maintained in the CADRAD, ICAR-Indian Veterinary Research Institute (IVRI) was revived in BHK-21 cells and dsRNA was extracted using phenol-chloroform RNA extraction method and lithium chloride precipitation. The extracted dsRNA was checked on polyacrylamide gel electrophoresis (PAGE) with silver nitrate staining for the presence of all the segments of the virus.

Amplification and cloning of NS3

cDNA synthesis was carried out using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Oligonucleotide primers targeting full-length NS3 gene were designed from the available GenBank sequences. Forward primer (GBK_S10_20F: AGATGAATTCATGCTATCCGGGC TGATC) and reverse primer (GBK_S10_709R: GACGTCGA CGTCAGGTTAATGGCATTTC) were flanked with *EcoRI* and *SalI* restriction endonucleases at 5' end respectively. Amplification of NS3 was done using DreamTaq DNA Polymerase (Thermo Scientific, USA). PCR amplification conditions were: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 10 min. Agarose gel electrophoresis of amplified product was done and the gel was purified using QIAquick® gel extraction kit (Qiagen, Hilden, Germany). The purified amplicon and pGBKT7-BD vector was double digested with *EcoRI* and *SalI* enzymes (NEB, USA). The digested products were ligated and the recombinant plasmid was initially transformed into a competent *E. coli* DH5α strain. The positive recombinant clones were confirmed by colony PCR, restriction enzyme digestion and nucleotide sequencing analysis.

pGBKT7-NS3 bait construction and its characterization

The sequenced confirmed pGBKT7-NS3 plasmid (100 ng) was retransformed to competent *Saccharomyces cerevisiae* (*S. cerevisiae*) Y2H Gold cells by lithium acetate transformation method (Gietz *et al*. 1995). The transformation mixture was diluted to 1/10 and 1/100 dilutions and 100 µl of each dilution was spread on SD/-Trp (SDO), SD/-Trp/ X-α-gal (SDO/X) and SD/-Trp/X-α-gal/AureobasidinA (SDO/X/A) plates (Clontech, USA) and incubated at 30°C for 3-5 days. The transformation was also performed simultaneously for positive (pGBKT7-53 + pGADT7-T), negative (pGBKT7-Lam + pGADT7-T) and bait control (empty pGBKT7-BD vector) and were also plated on SDO, SDO/X, SDO/X/A and SD/-Trp/-Leu/X-α-gal /AureobasidinA (DDO/X/A). The growth of the colonies was recorded after 3-5 days and compared with that of controls to check the presence of toxicity and auto-activation.

For expression, a single isolated colony of the pGBKT7-NS3, pGBKT7-53 (positive control) and Y2H Gold cells (negative control) were grown to an OD_{600} 0.4-0.6 with shaking (220 rpm) at 30°C. The cultures were centrifuged at 1000x g for 5 min at 4°C, washed with 50 ml of ice-cold water and were immediately frozen in liquid nitrogen (LN_2). The yeast protein extracts were prepared from the cell pellets following the TCA method according to the *Yeast Protocols Handbook* (Clontech). The extracted proteins were resolved on 12.5% sodium dodecyl sulfate polyacrylamide (SDS) gel and subsequently transferred to the nitrocellulose membrane (NCM). The membrane was blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline-Tween (TBST, 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) overnight at 4°C. Afterward, the membrane was incubated with primary mouse anti-c-myc monoclonal antibody (Clontech, USA) at 1:500 dilution for 1 hour at RT, followed by incubation with horseradish peroxidase- (HRP-) labeled goat anti-mouse IgG antibody (SantaCruz, USA) at 1:1000 dilution. The membrane was washed three times between each step with TBST and finally visualized by developing with a 3,3'-diaminobenzidine (DAB)-substrate solution.

Forward library screening

The generated pGBKT7-NS3 bait was mated with ovine cDNA library (available at MBL, IVRI) to screen the host protein interaction with NS3 protein of BTV. Briefly, the overnight grown culture of the bait in SD/-Trp medium was centrifuged and re-suspended in SD/-Trp medium to a cell density >$1x10^8$ cells/ml. The re-suspended bait culture was mixed with a 1 ml cDNA library in a 2 L sterile flask. 45 ml of 2X YPDA medium was added to it and incubated at 30°C with minimum shaking. Zygote formation was checked after 20 h under a phase-contrast microscope (40X). Thereupon the mated culture was serially diluted to 1/10, 1/100, 1/1000 and 1/10000 dilutions and 100 µl of each dilution were plated on SDO, SD/-Leu (SDO) and SD/-Leu/-Trp (DDO) agar plates. The remainder culture was plated on 150 mm (DDO/X/A) agar plates (200 µl per plate). Plates were incubated at 30°C for 3-5 days and mating parameters including the number of independent clones screened, the viability of prey, bait, diploids and efficiency of mating were estimated.

RESULTS AND DISCUSSION

Construction and characterization of bait

BHK-21 cells have shown cytopathic effect (CPE) after 72 h of infection with BTV-10. The characteristic CPE of BTV *i.e.* cell rounding, cell lysis and detachment has been observed (Fig 1) (Clavijo *et al*. 2000). The dsRNA was extracted and all the 10 segments were visible clearly on PAGE by silver

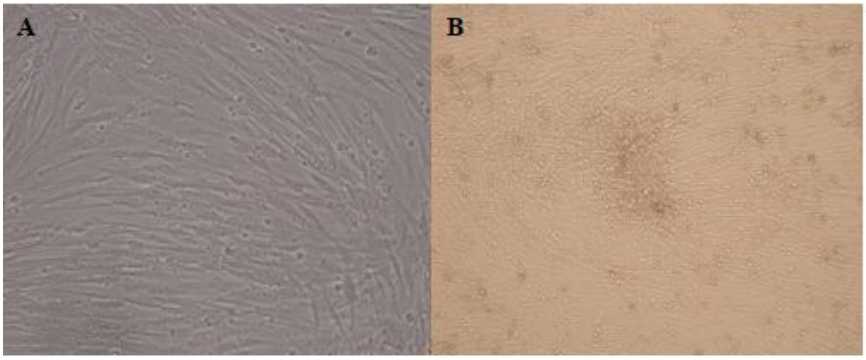

Fig 1: Cytopathic effect of BTV 10 on BHK-21 cells. (A) Uninfected BHK-21 control cells (72 h); (B) Infected BHK-21 cells, 72 h post-infection, showing cell rounding and rupture.

nitrate staining (Fig 2). The cDNA was synthesized and the full-length NS3 coding region of ~690 bp was amplified (Fig 3A). The NS3 amplicon was successfully double digested and cloned into the pGBKT7-BD vector as verified by colony PCR (Fig. 3B), restriction enzyme digestion (Fig 3C) and

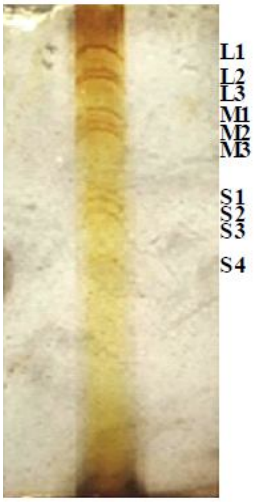

Fig 2: BTV dsRNA segments- silver nitrate staining.

nucleotide sequencing. Upon confirmation, the positive recombinant plasmid was retransformed into competent *S. cerevisiae* Y2H Gold cells. The colonies of pGBKT7-NS3 and positive control were equivalent in size indicating the absence of toxicity. Further, pGBKT7-NS3 bait gave only white colonies on both SDO, SDO/X plates and no growth was seen on SDO/X/A plates indicating NS3 does not autonomously activate the reporter gene (Fig 4) (Table 1). The constructed pGBKT7-NS3 bait was well expressed in yeast and a band of expected size ~46 kDa was detected by mouse anti-c-myc monoclonal antibody (Fig 5).

Toxic and self-activating bait yield a high frequency of false-positive interactions. Similarly, non-expressing or over-expressing bait also results in false-positive and non-specific interactions (Serebriiskii and Golemis, 2001). Qualification of all these parameters by bait is of at most importance to minimalize the chances of false-positive interactions with prey proteins (Brückner *et al*. 2009). The results imply that the constructed bait has qualified all the parameters and can be used as bait in Y2H to screen its interacting host proteins.

Library screening for interacting proteins

The mating between pGBKT7-NS3 bait and prey library was performed and grew on SDO (both SD/-Trp and SD/-Leu),

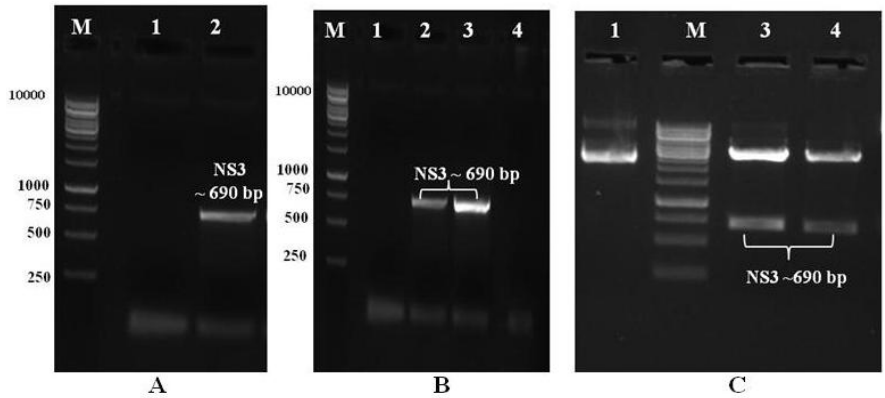

Fig 3 (A): PCR amplification of full length NS3 gene. Lane M: GeneRuler 1kb DNA ladder; Lane 1: Negative control; Lane 2: NS3 PCR amplicon. **(B)** Colony PCR of pGBKT7-NS3 colonies. Lane M: GeneRuler 1kb DNA ladder; Lane 1: no amplification; Lane 2,3: NS3 amplicon; Lane 4: Negative control. **(C)** RE digestion analysis. Lane M: GeneRuler 1kb DNA ladder; Lane 1: Undigested pGBKT7-BD plasmid; Lane 2, 3: pGBKT7-BD plasmid showing release of NS3 (~690 bp).

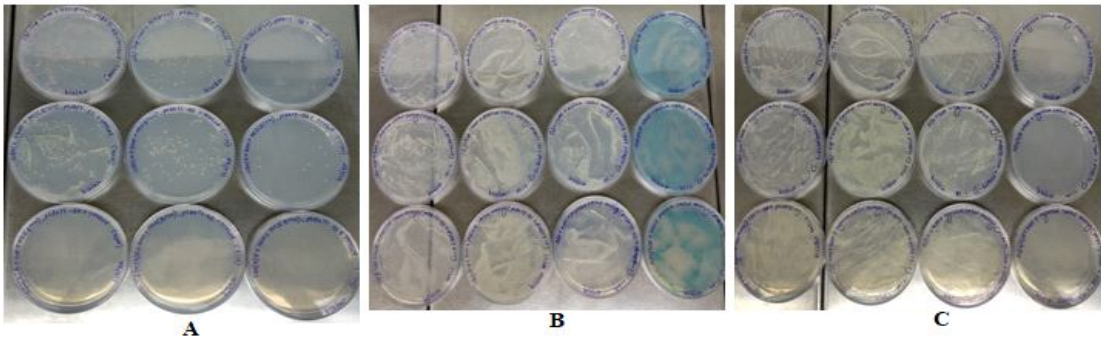

Fig 4: Autoactivation and toxicity analysis. **Panel A:** pGBKT7-NS3 bait- colonies in SD/-Trp, SD/-Trp/X agar plates but no colonies in SD/-Trp/X/A agar plates; **Panel B**: Positive control showing blue colonies on DDO/X/A agar plates; **Panel C**: Negative control showing colonies in SD/-Trp, SD/-Trp/X agar plates but no colonies in SD/-Trp/X/A and DDO/X/A agar plates.

Table 1: Tesing bait for autoactivation and toxicity.

Sampple	Selective agar plates	Presence of colonies	Colony colour
Bait autoactivation test	SDO	Yes	White
Bait autoactivation test	SDO/X	Yes	White
Bait autoactivation test	SDO/X/A	No	N/A
Positive control test	DDO/X/A	Yes	Blue

DDO and DDO/X/A plates. The viability of prey, bait and mating diploids was calculated from the colonies developed on SDO and DDO (Table 2). Approximately two million clones were screened and a mating efficiency of 3% was achieved. However, no blue colonies grew on DDO/X/A plates indicating the absence of an interacting proteins. The experiment was repeated but, similar results were obtained. As there are only two variables, bait and prey, to verify which one failed to perform, the mating was conducted using BTV NS4 bait since changing the bait is much easier and cost effective than changing the prey library (Brückner *et al*., 2009). The mating between BTV NS4 bait and the same library resulted in several interaction hits, indicating that the problem was with the NS3 bait only. In Y2H some full-length proteins do not yield interactions despite good expression as bait or prey. Full-length proteins fail to perform might be due to incorrect folding in yeast, precluding interaction with its partner, or due to their biology (Galletta and Rusan, 2015). On thorough analysis, we observed the presence of two transmembrane domains (TM) in our NS3 sequence. The position of TM domains were located from amino acids 122 to 139 and amino acids 166 to 182 using Phobius (Käll *et al*. 2004) and TMPred software (Hofmann and Stoffel, 1993) (Fig 6).

Previously, it has been reported that the expression of full-length NS3 protein is difficult in the prokaryotic system. Mutation analysis study has confirmed that the TM domains are responsible to produce this cytotoxicity in prokaryotic hosts (Chacko *et al*. 2015; Mohanty *et al*. 2016). The membrane association and glycosylation of NS3 protein of Ibaraki virus, a member of orbivirus genus have also been documented to have a cytotoxic effect (Urata *et al*. 2016). In addition to this, TM domain carrying NS3 protein of African

Table 2: Estimation of mating parameters.

Parameter	NS3-ovine library mating
Viabilty of prey library	1×10^{7} cfu/ml
Viabilty of bait	3×10^{8} cfu/ml
Viabilty of diploids	3×10^{5} cfu/ml
Number of clones screened	2×10^{6} clones
Mating efficiency	3%

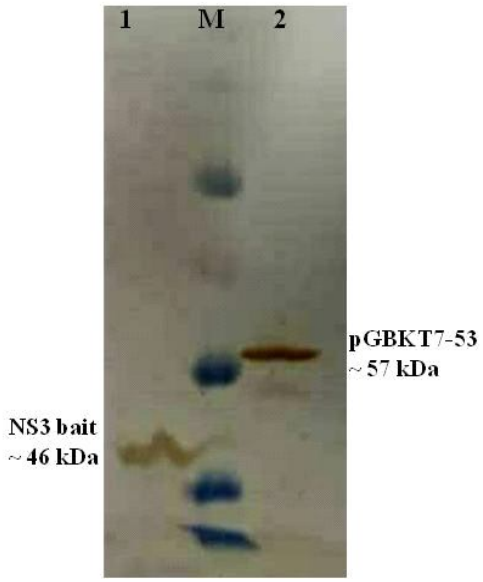

Fig 5: Western blot of expressed bait with c-myc mouse monoclonal antibody. Lane-M:Protein marker; Lane 1: pGBKT7-NS3 bait lysate showing recombinant NS3 protein(~46 kDa); Lane 2: pGBKT7-53 positive control (~57 kDa).

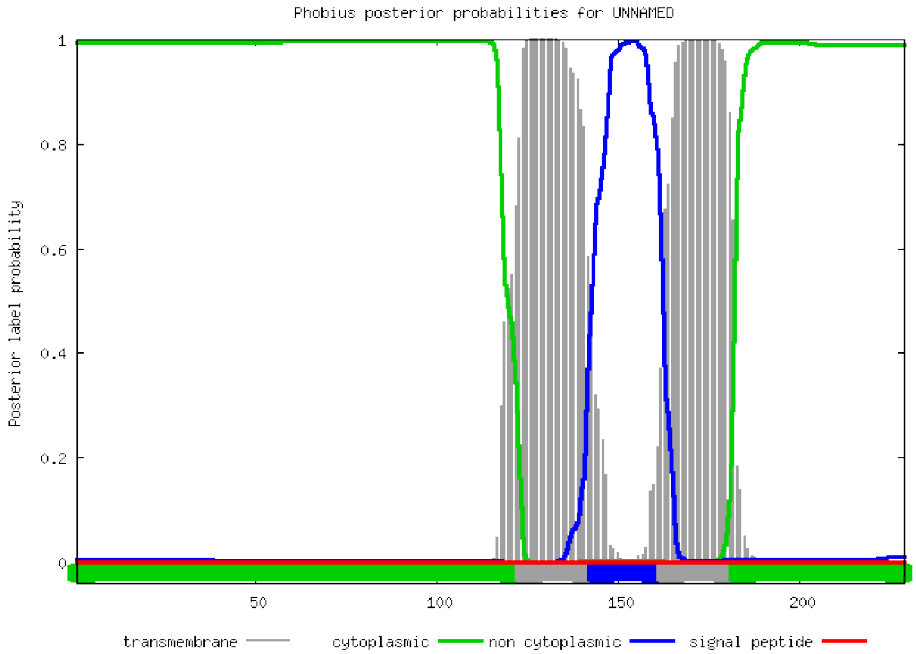

Fig 6: Full-length NS3 protein of BTV 10: showing two transmembrane domain (TM1 and TM2).

horse sickness virus (AHSV), another orbivirus found to produce cytotoxic effect on protein expression in the baculovirus system (van Staden *et al*. 1995; van Niekerk *et al*. 2001). NS3 protein from orbiviruses has been reported to produce cytotoxicity in mammalian and insects cells too (van Staden *et al*. 1995; Wirblich *et al*. 2006). For producing cytotoxicity NS3 requires the membrane association as well as the presence of the transmembrane domains (Han and Harty, 2004). Literature suggests that the structure and distribution of the TM domains within NS3 protein control the expression of cytotoxicity it produce. Thus it can be hypothesized that the TM domains in the full-length NS3 protein were interfering with PPI studies in the Y2H system. Mutations causing alterations in the TM domains or deletion of these TM domains reduce the cytotoxicity of the protein (van Niekerk *et al*. 2001; Huismans *et al*. 2004; Mohanty *et al*. 2016). The studies available on BTV NS3-host PPI using Y2H system has reported the p11, a light chain component of the Calpactin complex and the serine/threonine-protein kinase B33 Raf (BRAF) as an interacting cellular factors (Beaton *et al*. 2002; Kundlacz *et al*. 2019). Among these, Beaton *et al* (2002) has mentioned the use of the truncated portions of NS3 only for Y2H study. The presence of TM domains in our bait sequence may also be producing some cytotoxicity during interaction with prey proteins and may be the reason for not getting the interactions upon mating. Thus, we redesigned our experiment to generate N-terminus and C-terminus truncated NS3 excluding the TM domains and we are in the process of performing it.

CONCLUSION

We here report the construction and characterization of pGBKT7-NS3 bait to study the BTV NS3 PPI. pGBKT7-NS3 bait was found to be well expressed and suitable to screen host-virus interaction. However, we could not found any interaction with the full-length NS3 protein owing to the presence of TM domains. Thus we hypothesize that the presence of TM domains interfere with the transcription of reporters in the Y2H system. For the future, we redesigned our study to include truncated NS3 as a bait, eliminating TM domains to screen host-virus PPI.

ACKNOWLEDGEMENT

The authors are grateful to the Indian Council of Agricultural Research and SERB for providing funding.

Funding information

This work was funded by the Science and Engineering Research Board (SERB) under the project ECR/2017/001106.

Conflict of interest statement

The authors declare no conflict of interest.

REFERENCES

Alkhamis, M.A., Aguilar-Vega, C., Fountain-Jones, N.M., Lin, K., Perez, A.M. and Sánchez-Vizcaíno, J.M. (2020). Global emergence and evolutionary dynamics of bluetongue virus. Scientific Reports 10 (1): 1-12.

Beaton, A.R., Rodriguez, J., Reddy, Y.K. and Roy, P. (2002). The membrane trafficking protein calpactin forms a complex with bluetongue virus protein NS3 and mediates virus release. Proceedings of the National Academy of Sciences. 99(20): 13154-13159.

Belbis, G., Bréard, E., Cordonnier, N., Moulin, V., Desprat, A., Sailleau, C., Viarouge, C., Doceul, V., Zientara, S. and Millemann, Y. (2013). Evidence of transplacental transmission of bluetongue virus serotype 8 in goats. Vet. Microbiol. 166(3-4): 394-404.

Belhouchet, M., Mohd Jaafar, F., Firth, A.E., Grimes, J.M., Mertens, P.P. and Attoui, H. (2011). Detection of a fourth orbivirus non-structural protein. PLoS One 6(10): e25697.

Brückner, A., Polge, C., Lentze, N., Auerbach, D. and Schlattner, U. (2009). Yeast two-hybrid, a powerful tool for systems biology. Int. J. Mol. Sci. 10(6): 2763-2788.

Bumbarov, V., Golender, N., Jenckel, M., Wernike, K., Beer, M., Khinich, E., Zalesky, O. and Erster, O. (2020). Characterization of bluetongue virus serotype 28. Transbound Emerg. Dis. 67(1):171-182.

Celma, C.C. and Roy, P. (2009). A viral nonstructural protein regulates bluetongue virus trafficking and release. J. Virol. 83(13): 6806-6816.

Chacko, N., Mohanty, N.N., Biswas, S.K., Chand, K., Yogisharadhya, R., Pandey, A.B., Mondal, B. and Shivachandra, S.B. (2015). A coiled-coil motif in non-structural protein 3 (NS3) of bluetongue virus forms an oligomer. Virus Genes. 51(2): 244-251.

Clavijo, A., Heckert, R.A., Dulac, G.C. and Afshar, A. (2000). Isolation and identification of bluetongue virus. J. Virol. Methods. 87(1-2):13-23.

Coetzee, P., Stokstad, M., Venter, E.H., Myrmel, M. and Van Vuuren, M. (2012). Bluetongue: A historical and epidemiological perspective with the emphasis on South Africa. Virology Journal. 9(1): 1-9.

Fields, S. and Song, O. (1989). A novel genetic system to detect protein-protein interactions. Nature. 340(6230): 245-246.

Galletta, B.J. and Rusan, N.M. (2015). A yeast two-hybrid approach for probing protein-protein interactions at the centrosome. Methods Cell Biol. 129: 251-277.

Gietz, R.D., Schiestl, R.H., Willems, A.R. and Woods, R.A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast. 11(4): 355-360.

Han, Z. and Harty, R.N. (2004). The NS3 protein of bluetongue virus exhibits viroporin-like properties. J. Biol. Chem. 279(41): 43092-43097.

Hoffman, K. and Stoffel, W. (1993). TMbase-A database of membran spsnning proteins segments. Biol Chem. Hopper-Seyler 347: 166

Huismans, H., Van Staden, V., Fick, W.C., Van Niekerk, M. and Meiring, T.L. (2004). A comparison of different orbivirus proteins that could affect virulence and pathogenesis. Vet Ital. 40(4): 417-425.

Käll, L., Krogh, A. and Sonnhammer, E.L. (2004). A combined transmembrane topology and signal peptide prediction method. Journal of Molecular Biology. 338(5): 1027-1036.

Kundlacz, C., Pourcelot, M., Fablet, A., Amaral Da Silva Moraes, R., Léger, T., Morlet, B., *et al* (2019). Novel function of bluetongue virus NS3 protein in regulation of the MAPK/ERK signaling pathway. J. Virol. 93(16): e00336-19.

Meiswinkel, R., Baldet, T., de Deken, R., Takken, W., Delécolle, J.C. and Mellor, P. S. (2008). The 2006 outbreak of bluetongue in northern Europe-the entomological perspective. Prev. Vet. Med. 87(1-2): 55-63.

Mellor, P.S. (1990). The replication of bluetongue virus in Culicoides vectors. Curr. Top Microbiol Immunol. 162: 143-161.

Mellor, P.S., Carpenter, S., Harrup, L., Baylis, M. and Mertens, P.P. (2008). Bluetongue in Europe and the Mediterranean Basin: History of occurrence prior to 2006. Prev. Vet. Med. 87(1-2): 4-20.

Modrow, S., Falke, D., Truyen, U. and Schaetzl, H. (2010). 3rd ed ed: Springer Spektrum.

Mohanty, N.N., Chacko, N., Biswas, S.K., Chand, K., Pandey, A.B., Mondal, B., Hemadri, D. and. Shivachandra, S.B (2016). Production of recombinant non-structural protein-3 hydrophobic domain deletion (NS3ΔHD) protein of bluetongue virus from prokaryotic expression system as an efficient diagnostic reagent. Biologicals. 44 (5): 352-359.

Rao, P.P., Hegde, N.R., Reddy, Y.N., Krishnajyothi, Y., Reddy, Y.V., Susmitha, B., Gollapalli, S.R., Putty, K. and Reddy, G.H. (2016). Epidemiology of Bluetongue in India. Transbound Emerg. Dis. 63(2): e151-164.

Ratinier, M., Caporale, M., Golder, M., Franzoni, G., Allan, K., Nunes, S.F., Armezzani, A., Bayoumy, A., Rixon, F., Shaw, A. and Palmarini, M. (2011). Identification and characterization of a novel non-structural protein of bluetongue virus. PLoS Pathog. 7(12): e1002477.

Rojas, J.M., Rodríguez-Martín, D., Martín, V. and Sevilla, N. (2019). Diagnosing bluetongue virus in domestic ruminants: Current perspectives. Vet. Med. (Auckl) 10:17-27.

Saminathan, M., Singh, K.P., Vineetha, S., Maity, M., Biswas, S.K., Manjunathareddy, G.B., Chauhan, H.C., Milton, A.A.P., Ramakrishnan, M.A. *et al.* (2020). Virological, immunological and pathological findings of transplacentally transmitted bluetongue virus serotype 1 in IFNAR1-blocked mice during early and mid gestation. Sci. Rep. 10(1): 2164.

Schwartz-Cornil, I., Mertens, P.P., Contreras, V., Hemati, B., Pascale, F., Bréard, E., Mellor, P.S., MacLachlan, N.J. and Zientara, S. (2008). Bluetongue virus: Virology, pathogenesis and immunity. Vet. Res. 39(5): 46.

Serebriiskii, I.G. and Golemis, E.A. (2001). Two-hybrid System and False Positives. In: Two-Hybrid Systems. Humana Press. pp. 123-134

Sperlova, A. and Zendulkova, D. (2011). Bluetongue: a review. Vet Med-Czech. 56(9): 430-452.

Sreenivasulu, D., Subba Rao, M.V., Reddy, Y.N. and Gard, G.P. (2004). Overview of bluetongue disease, viruses, vectors, surveillance and unique features: The Indian sub-continent and adjacent regions. Vet Ital. 40(3): 73-77.

Stewart, M., Hardy, A., Barry, G., Pinto, R.M., Caporale, M., Melzi, E., Hughes, J., Taggart, A., Janowicz, A., Varela, M., Ratinier, M. and Palmarini, M. (2015). Characterization of a second open reading frame in genome segment 10 of bluetongue virus. J. Gen. Virol. 96(11): 3280-3293.

Urata, M., Watanabe, R. and Iwata, H. (2016). The effect of glycosylation on cytotoxicity of Ibaraki virus nonstructural protein NS3. J. Vet. Med. Sci. 77(12): 1611-1616.

van Niekerk, M., Smit, C.C., Fick, W.C., van Staden, V. and Huismans, H. (2001). Membrane association of African horsesickness virus nonstructural protein NS3 determines its cytotoxicity. Virology. 279(2): 499-508.

van Staden, V., Stoltz, M.A. and Huismans, H. (1995). Expression of nonstructural protein NS3 of African horsesickness virus (AHSV): Evidence for a cytotoxic effect of NS3 in insect cells and characterization of the gene products in AHSV infected Vero cells. Arch Virol. 140(2): 289-306.

Wirblich, C., Bhattacharya, B. and Roy, P. (2006). Nonstructural protein 3 of bluetongue virus assists virus release by recruiting ESCRT-I protein Tsg101. J. Virol. 80(1): 460-473.

Wu, X., Chen, S.Y., Iwata, H., Compans, R.W. and Roy, P. (1992). Multiple glycoproteins synthesized by the smallest RNA segment (S10) of bluetongue virus. J. Virol. 66(12): 7104-7112.

Yang, H., Gu, W., Li, Z., Zhang, L., Liao, D., Song, J. and Li, H. (2020). Novel Putative Bluetongue Virus Serotype 29 Isolated from Inapparently Infected Goat in Xinjiang of China. Transbound Emerg Dis. 68(4): 2543-2555.